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INCLUSIVE DATES 15 February 1962 TO 14 February 1963

SUBJECT OF INVESTIGATION

Studies On The Anti-Viral Chemotherapeutic Drugs On Neurotropic Viruses, Particulary Japanese B Encephalitis And Poliomyelitis

RESPONSIBLE INVESTIGATOR

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The abstract of Final Report No.2

On Contract No. DA-92-557-FEC-34625

The purpose of this work is to find effective antiviral agents on poliomyelitis and Japanese B Enecephalitis viruses, and the findings obatined in this work will be a milestone to find antiviral compounds on any other small animal viruses. For this purpose, 107 compounds were synthesized and screened as to their effect. Among them, it was found that compound. No. 799, 4-Amino-6-(N-morpholinyl)-s-triazine-2-carboxyguanidide, was highly inhibitory on the multipication of polio, ECHO and coxsackie viruses, and the site of action of compound. No.799 must be in intracellular site. In addition of the above findings, it was also found that compound.No.799 showed the effect on influenza in mice. In the experiments to obtain some biological high molecular sunstances like interferon, the responsible investigator found that the culture fluid of cells without any infection of virus produced an inhibitory agent on the development of plaque of all three types of poliomyelitis virus. This substance was quite different from any known inhibitory natural substances such as Interferon, viral inhibitory factor reported by Drs. Enders and Ho, Holland's receptor, neutralizing immune serum, nonspecific inhibitor in serum like α and β inhibitors and VIF of Dr.Nagano. The findings of both compound. No.799 and the viral inhibitory substance found in normal culture fluid will be a key to open the door of antiviral chemotherapy.

FINAL REPORT NO.2 ON

CONTRACT NO. DA-92-557-FEC-34625

INCLUSIVE DATES 15 FEBRUARY 1962 TO 14 FEBRUARY 1963

Subject Of Investigation

Studies On The Anti-viral Chemotherapeutic Drugs On Neurotropic Viruses, Particularly Japanese B Encephalitis And Poliomyelitis.

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Contents.

1. A Statement of the Problem.

The purpose of this investigation is to find antiviral drugs on such neurotropic viruses as Japanese B Encephalitis and poliomyelitis, and if some effective antiviral agents are found in this investigation, the findings will be a valuable milestone to obtain highly effective inhibitory substances against any other small animal viruses. Upto the present day, a lot of reports as to antiviral agents have so far been published by many research workers, but no compound has been recognized as an antiviral chemotherapeutic drug. About two years ago, ABOB and Xenalamine were reported as effective agents on influenza virus in mice by Dr.Melander and Dr.Magrassi, but the effect of both compounds was not confirmed by the reexamination of our research group and many other research groups in not only Japan and The States, but many countries in the world. In other words, it may safely be said that any effective antiviral drugs has not been found yet in the world.

To find effective antiviral agents, guanido group containing compounds and the homolugues of the bases of uncleic acid such as pyrimidine-, triazine-, purine- derivatives and so on, should be noticed by the responsible investigator as the most promising way, and as another important research program, the search to find antiviral natural substance like Interferon or Interferon like substances must be noticed.

During the past one year, the investigation along the above research planning has been carried out under the

contract with U.S.Army R. and D. Group (Far East), and some important fundamental knowledges to conceive new types of compounds were obtained as described in the following sections.

It is a great privilege for the responsible investigator that the outline of the research work carried out during the past one year is described here.

2. Analysis of the Problem.

In this investigation, the following two important points are included.

A. The nature of the viral multiplication in host cells and the biochemical character of the degeneration of cells infected with virus have not been settled yet. To solve these points, a lot of research workers have carried out the experiments, but most of the experiments are limited to the investigation of time-course of biosyntheses of both protein and uncleic acid in the host cells infected with virus, and any specific pattern of cell-metabolism essential for viral replication has not been found. As described in the final report No. 1 of the contract, the responsible investigator found that polio virus acquired clear resistance against the antiviral effect of guanidine if the virus showed the reproduction in the presence of guanidine. The minute analysis of the nature of the development of guanidine resestant polio virus will be a tool to solve the essential pathway for the replication of polio virus, and the fundamental knowledges obtained from such an experiment will be able to be expanded to the search as to the cell-

metabolism necessary for the reproduction of any other small animal virus, and those knowledges will be very much valuable milestone to conceive and synthesize new types of c compounds.

B. To find highly effective antiviral agents, the following research programs were planned, because the responsible investigator has already found the effect of guanidine on polio virus and the effect of 5-Butyluracil on Japanese B Encephalitis and it was found that the effect of those compounds may be due to the inhibition of the biosynthesis of viral uncleic acid and/or the process of viral maturation.

(1) Synthesis of Compounds.

a; Guanido group containing compounds.

b; Pyrimidine derivatives.

c; Triazine derivatives.

d; Purine derivatives.

e; Thiourea derivatives.

f; Any other homolugues of bases of nucleic acid.

(2) The Search to Find Biological Substances Inhibitory on the Multiplication of Virus.

From a lot of reports concerning the mode of action of Interferon or Interferon like substance, it was suggested by Isaacs, Lockhart and many other workers that Interferon should show the effect due to the inhibition of the biosynthesis of viral infectious uncleic acid in the host cells infected with virus. The responsible investigator took an interest in such mechanism

of Interferon or Interferon like substance, but the inhibitory effect of those substances seemed too weak to be recognized as highly effective antiviral agents. Thus, the responsible investigator carried out the search to find other experimental system producing viral inhibitory natural substances.

3. Outline of Experimental Procedures.

All compounds were synthesized chemically except viral inhibitory natural substances and they were identified with elementary analysis and in some cases confirmed with U.V. or I.R. spectrophotometry. Then, all synthesized compounds were screened as to their antiviral effect as follows;

(1) First Screening Test:

Tissue culture method was employed in polio and Japanese B Encephalitis. For polio virus, the Mahoney strain and for Japanese B Enecephalitis, the Nakayama strain were employed. As host cells, both of HeLa and Hep.No.2 cells were used. After the determination of toxicity of tested compounds on host cells, the inhibitory effect of the compounds was examined. 100 $TCID_{50}$ of a viral material was inoculated soon after the solution of a tested compound was added into tubes in which the monosheet of host cells had been established, and then they were incubated at 37℃ for 7 days in polio experiments and for 3 days in Japanese B Encephalitis experiments. In Japanese B Encephalitis experiments, the culture fluid was inoculated intracereberally into mice, then, these

mice were observed for two weeks. To be recognized as an effective compound the multiplication of virus in host cells must be completely suppressed.

(2) Second Step of Experiments:

If an effective compound is found from the first screening test, it will be investigated the antiviral nature in more detail. For example, the influences of the agent on the viral absorption and the penetration into host cells, the influences of the agent on the viral release from host cells, the viral direct inactivating effect, the inhibitory effect of the agent on the single multiplication cycle of virus, the antiviral spectrum of the agent and so on. For the experiments to investigate the antiviral spectrum of a compound, as viral materials the following viruses were employed. polio(type-1, type-2, type-3), ECHO-6 and ECHO-9, Coxsackie A-9, Coxsackie B-5, adeno virus(from type-1 to type-7), vaccinia, influenza A-2. All these viral materials except influenza were studied by using tissue culture method, and for the examination of anti-influenza effect mice were employed. In addition of the above fundamental studies of the inhibitory effect of a compound, the effectiveness of a compound was further, in detail , investigated to obtain knowledges for the practical application of the compound. In other words, the minimum effective dosis of the compound, the determination of chemotherapeutic index, the relationship between viral inoculum size and the inhibitory effect of the compound and so on.

4. Experimental Results.

During the past one year, from 15 February 1962 to 14 February 1963, the following experimental results are obtained.

A. Syntheses of compounds and screening test of them.

The following 107 compounds were synthesized and screened as to their effect.

Compound No.	Chemical name.
1	N'-(β-Aminoethyl)-guanidine sulfate
2	Trimethylenediguanidine sulfate
3	Ethylenediguanidine ditoluenesulfonamide
4	Ethyleneguanidine hydrobromide
5	Pentamethylenediguanidine sulfate
6	Tetramethylenediguanidine sulfate
7	2-Amino-6-hydroxy-8-ethylthiopurine
8	2,4-Diamino-5-actamino-6-hydroxypurine
9	2-Hydrazino-4,6-diamino-s-triazine hydrochloride
10	4-Methylthiazol-2-yl-guanidine hydrochloride
11	4-Hydroxybenzenesulfonyl guanidine
12	1-(p-Tolyl)-3-(4,5-dioxo-2-imidazolidinylidene)guanidine
13	Isopropylnitroguanidine
14	Nitroguanidine
15	2,4-Diamino-5-propioamino-6-hydroxypyrimidine
16	2-Mercapto-4-amino-5-acetamino-6-hydroxypyrimidine
17	2,4-Diamino-6-propyoamino-6-hydroxypyrimidine
18	Toluenesulfonylmethylguanidine
19	Toluenesulfonylethylguanidine
20	p-Acetaminophenylglyoxal hydrate

21 2-Mercapto-4-amino-5-propioamino-6-hydroxyprimidine

22 2-(N^3-Cyclohexylguanidyl)-hydroxy-6-methyl-pyrimidine

23 2-(N^3-Benzylguanidyl)-4-hydroxy-6-methylpyrimidine

24 2-(N^3-Hexylguanidyl)-4-hydroxy-6-methylpyrimidine

25 2-(N^3-n-Propylguanidyl)-4-hydroxy-6-methyl-pyrimidine

26 Toluenesulfonylbutylguanidine

27 Toluenesulfonylpropylguanidine

28 2-Morpholinyl-4-amino-s-triazine-6-carboxyguanidide

29 2-Isopropylamino-4-amino-6-carboanilide-s-triazine

30 4-Amino-6-(N-morpholinyl)-s-triazine-2-carboxy-molpholide

31 4-Amino-6-(N-morpholinyl)-s-triazine-2-carboxy-peridide

32 4-Amino-6-(N-morpholinyl)-s-triazine-2-carboxy-pyrrolidide

33 4-Amino-6-(N-morpholinyl)-s-triazine-2-cartanilide

34 4-Amino-6-isopropylamino-s-triazine-2-carboxy-piperidide

35 4-Amino-6-isopropylamino-s-triazine-2-carboxy-morpholide

36 4-Amino-6-isopropylamino-s-triazine-2-carboxy-pyrrolidide

37 4-Amino-6-isopropylamino-s-triazine-2-carboxy-isopropylamide

38 4-Amino-6-isopropylamino-s-triazine-2-carboxy-guanidide

39 4-Amino-6-cyclohexylamino-s-triazine-2-carboxy-pyrrolidide

40 4-Amino-6-cyclohexylamino-s-triazine-2-carbanilide

41 4-Amino-6-cyclohexylamino-s-triazine-2-carboxypiperidide

42 4-Amino-6-cyclohexylamino-s-triazine-2-carboxymorpholide

43 4-Amino-6-cyclohexylamino-s-triazine-2-carboxyisopropylamide

44 4-Amino-6-(p-methoxyphenyl)amino-s-triazine-2-carboxyisopropylamide

45 4-Amino-6-(p-methoxyphenyl)amino-s-triazine-2-carboxyguanidide

46 4-Amino-6-(p-methoxyphenyl)amino-s-triazine-2-carboxypyrrolidide

47 4-Amino-6-(p-methoxyphenyl)amino-s-triazine-2-carboxypiperidide

48 4-Amino-6-(p-toluidino)-s-triazine-2-carboxymorpholide

49 4-Amino-6-(p-toluidino)-s-triazine-2-carboxypiperidide

50 4-Amino-6-(p-toluidino)-s-triazine-2-carboxyisopropylamide

51 4-Amino-6-(p-toluidino)-s-triazine-2-carbanilide

52 4-Amino-6-(p-toluidino)-s-triazine-2-carboxypyrrolidide

53 Nitrosoguanidine

54 Diethyl-p-guanidylacetaminomalonate

55 Azodicarbamidine nitrate

56 1,1-Anhydrobis-(β-hydroxyethyl)-2-ethylthiourea

57 N′,N′-Anhydrobis-(β-hydroxyethyl)-N^3-methylguanidine sulfate

58 1,2-Diphenyl-1,2-di-N,N-dimethylaminoethane methyliodide

59 p-(p′-Nitrobenzenesulfonyl)-aminophenol

60 p-Acetaminophenyldimethylaminoethylether

61 2-Methyl-4-hydroxy-5-aminomethylpyrimidine hydrochloride

62 2-Methyl-4-amino-5-(5-methylguanidinomethyl)-pyrimidine sulfate

63 2-Methyl-4-hydroxy-5-guanidinomethylpyrimidine sulfate

63 2-Methyl-4 , ,6-dichloro-5-isopropylpyrimidine

64 2-Methyl-4-methylamino-6-chloropyrimidine hydrochloride

65 2-Methyl-4,6-dihydroxy-5-isopropylpyrimidine

66 2-Methyl-4-methylamino-5-isopropyl-6-chloropyrimidine

67 2-Methyl-4,6-dichloropyrimidine

68 2-Methyl-4,6-dihydrooxypyrimidine

69 N,N-Diguanidinopropylamine

70 3-N,N-Dimethylaminopropylguanidine

71 N-Guanidinoethyl-4-ethylpiperadine sulfate

72 N-Guanidinoethyl-4-methylpiperadine sulfate

73 1,1-Anhydrobis-(β-hydroxyethyl)-2-benzoylthiourea

74 1,1-Anhydrobis-(β-hydroxyethyl)-2-propylthiourea

75 N′,N′-Anhydrobis-(β-hydroxyethyl)-2-methylthiourea

76 1,1-Anhydrobis-(β-hydroxyethyl)-2-methylthiourea

77 Hydrazodicarbamidine dinitrate

78 p-Hydroxypenylglyoxal-2-pyrimidinohydrazone

79 p-Hydroxyphenylglyoxal-(3-methyl-5-isopropyoxy-2-pyrmidinohydrazone)

80 p-Hydroxyphenylglyoxal-(2-amino-4-methyl-6-pyrimidino-hydrazone)

81 p-Hydroxyphenylglyoxal-(3-methyl-5-ethoxy-2-pyrimidinohydrazone)

82 Formoguanamine

83 4-Amino-6-(N-morpholinyl)-s-triazine-2-carboxylic acid

84 1-Isopropyl-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

85 1-Cyclohexyl-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

86 1-Ethyl-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

87 1-Hexyl-3-(4,5-dioxo-2-imidazolidinymidene)-guanidine

88 1-(4,5-dioxo-2-imidazolidinylidene)-guanidine

89 1-(N',N'-anhydro-bis(β-hydroxyethyl))-3-(4,5-dioxo-2-imidazolidinylidine)-guanidine

90 1-Benzyl-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

91 1-(p-Methoxyphenyl)-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

92 1-Butyl-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

93 1-Propyl-3-(4,5-dioxo-2-imidazolidinylidene)-guanidine

94 3-Mercapto-5-hydroxy-6-(2,4-dichlorobenzyl)-1,2,4-triazine

95 β-(3,4-Diethoxyphenyl)-pyruvic acid-thiosemicarbazone

96 β-(p-Bromophenyl)-pyrvic acid-thiosemicarbazone

97 β-(2,4-Dichlorophenyl)-pyrvic acid-thiosemicarbazone

98 p-(p'-Hydroxybenzenesulfonylamino)-benzoic acid

99 p-Dimethylaminocarbamide anilide

100 p-Dimethylaminoacrbamide phenol

101 p-Dimethylaminocarboxyacetanilide

102 p-(p'-Aminobenzenesulfonic amino)-benzoic acid

103 p-(p'-Aminobenzenesulfonylamino)-benzamide sulfate

104 p-(p'-Hydroxybenzenesulfonylamino)-phenol

105 β-Dimethylamino-ethyl-p-nitrophenyl-ether maleate

106 p-(p'-Acetaminobenzenesulfonylamino)-benzamide

107 p-(p'-Acetaminobenzenesulfonylamino)-benzoic acid

The above 107 compounds were synthesized during the contract period, from 15 February 1962 to 14 February 1963, and all of these compounds were screened as to their effect. Among these 107 compounds, the two compounds of 2-Morpholinyl-4-amino-s-triazine-6-carboxyguanidide and 2-Isopropylamino-4-amino-6-carboamidinamide-s-triazine were noticed as the most promising compounds. The latter compounds, however, was used up on the way of the investigation, and so the antiviral effect of the former compound has been investigation in more detail. The latter compound will be investigated after the larger amount of it is prepared. Any of the other 105 compounds, did not show the effect so clearly as Guanidine did.

B. Antiviral Effect of 2-Morpholinyl-4-amino-s-triazine-6-carboxy-guanidide(No. 799).

2-Morpholinyl-4-amino-s-triazine-6-carboxyguanidide is called No. 799 in the Institute of the responsible investigator.

First of all, the toxicity of No. 799 on both of the KB and the Hep. No. 2 cells. The Max. nontoxic dosis of this compound on both host cells was M/500, and LD_{50} of this compound in mice was 1500mg/kg in the intraperitoneal administration. From these results, it may be said that No. 799 is less toxic.

(a) Antiviral effect of No. 799 in tissue culture system

10^{-3}M of this compound was added into assay tubes in which the monosheet of emplyed cells had been established,

and in minutes, various dilutions of a tested material were inoculated into these tubes, then the tubes were incubated at 37℃, and from the daily observation the $TCID_{50}$ of both the control and the treated groups was calculated. The experimental results are shown in Table 1.

Table 1

Antiviral Effect of No.799

Viruses	$TCID_{50}(-Log_{10})$ Control	Treated
Polio		
Mahoney	7	3
MEF_1	7	3.5
Saukett	7.5	2.5
ECHO		
Type-6	5.5	3.5
Type-9	5	5.5
Coxsackie		
A-9	3.5	2.5
B-5	7.5	6.5
Adeno		
from type-1 to type-7	5.5	5.5
Vaccinia		
DV-96	5.0	4.5
Japanese B Encephalitis		
Nakayama	3.5	3.5
Measles		
Edmonstan	7.5	3.0

As can be seen in Table 1, No. 799 was effective on both entero virus such as polio, ECHO-6, coxsackie A-9 and B-6 and

measles virus, but not on such DNA containing viruses as adeno and vaccinia viruses. From the above results, it was suggested that No.799 may be effective on not only entero virus and measles but some other RNA containing viruses. Thus, the effect of this compound on influenza in mice was investigated. In addition of the examination of the anti-influenzal effect of No.799, the effect of this compound on Japanese B Encephalitis in mice was investigated to confirm the above negative results in the experiments using tissue culture system. Influenza problem is not included in the subject of the contract, but the responsible investigator carried out the investigation as to the effect of No.799 on this virus because of the similarity of this virus to both polio and Japanese B Encephalitis virus in point of view of quality of the nucleic acid core and the importance of influenza problem.

Various dilutions of Tokyo-57/Adachi/2 strain of influenza virus were inoculated intranasally into mice, and soon later, the solution of No.799 was injected intraperitoneally in a dose of 500mg/kg/day during 4 days. These mice were observed for two weeks after the viral inoculation. At the end of the observation-days, both of LD_{50} and CD_{50} (consolidation $dosis_{50}$) were recorded.

The experimental results are shown in Table 2.

Table 2.

Effect of No.799 on Influenza in Mice

Viral dilutions inoculated	Administered time after viral inoculation	Control	Results Treated
10^{-1}		5/5 (3.6)	5/5※ (4.7)※※
10^{-2}	0,24,48,72 and 96	5/5 (6.7)	5/5 (8.3)
10^{-3}		5/5 (6.8)	5/5 (9.3)
10^{-4}		3/5	1/5
10^{-5}		0/5 (1.2)	0/5 ※※※ (0.5)
10^{-6}		0/5 (1)	0/5 (0.3)
10^{-7}		0/5 (0.6)	0/5 (0)
LD_{50}		$10^{-4.16}$	$10^{-3.62}$
CD_{50}		10^{-7}	$10^{-5.75}$

※ Died mice/Total used mice.

※※ Average life days of died mice.

※※※ Consolidation score of survived mice.

The results shown in Table 2 suggest that No.799 may be effective on influenza in mice. The problem of chemotherapy of respiratory viruses is also one of the most important problems in virology, but any highly effective chemotherapeutic drug has not been found yet. Thus, the responsible investigator hope that the discovery of No.799 could be a milestone of this research field.

In the experiments to confirm the effect of No.799 on

Japanese B Encephalitis in mice, $5XLD_{50}$ of the Nakayama strain was inoculated intraperitoneally into mice, then 72 hours later, $1/3LD_{50}$ of No. 799 (500mg/kg) was injected intraperitoneally into these mice. During two weeks after the viral inoculation, the appearance of systoms and the death of the mice were daily observed. But no difference between the mortality and the average survival days of the control and those of the treated group was observed. From the results, it may be said that No. 799 was ineffective on Japanese B Encephalitis.

(b) Site of action of No. 799

The site of action of No. 799 on both of polio and measles viruses was investigated, and the following results were obtained.

1. No. 799 does not inactivate the infectivity of virus.
2. No. 799 does not inhibit the viral adsorption onto host cells.
3. No. 799 does not inhibit the viral release from host cells.
4. No. 799 inhibits the intracellular multiplication of virus when it was added into assay tubes at the latent period of viral reproduction.

$10^{4.5}$ $TCID_{50}$ of the Mahoney strain of polio virus was added into assay tubes in which the monosheet of host cells had been established, and these tubes were incubated at 37℃ for one hour. Then, the medium was removed from the tubes, and the cells were washed three times with phosphate buffered saline to remove unadsorbed virus, and then 10^{-3}M of No. 799 was added into the tubes. In the control group, the

same amount of phosphate buffered saline(PBS) was added in instead of No.799. After the cultivation for 18 hours, the viral amount of both of culture fluid and intracellular fraction was estimated. The experimental results are shown in Table 3.

Table 3

Inhibition of No.799 on the Reproduction of Mahoney Strain.

Viral Inoculum Size	Dosis of No. 799	$TCID_{50}$ ($-Log_{10}$) Extracellular C	Extracellular T	Intracellular C	Intracellular T
$10^{4.5}$ $TCID_{50}$	10^{-3}M	5.5	1	7.5	3

As can be seen in Table 3, No.799 inhibited clearly the intracellular multiplication when it was added at the viral replication stage. From the results, it may be said that the site of action of No.799 must be in intracellular site.

C. Development of the resistant poliomyelitis virus to Guanidine and No.799.

As described in the final report No.1 of this contract, polio virus showed the resistance to the effect of guanidine after the passage in the guanidine containing maintenance medium. In the field of chemotherapy, the mode of action of a chemotherapeutic drug has often been made clear from the search as to the mechanism of the development of a resistant microorganism to the drug. Thus, the time course of the development of the guanidine resistant polio virus, the development of the No.799 resistant polio virus and the cross-resistance between the guanidine resistant virus and the No.799 resistant one were investigated.

(a) Development of No.799 resistant polio virus.

As described in the section-B of this report, No.799 can inhibit the growth of $10^{3.5}$-10^{5} X $TCID_{50}$ of polio virus, but the progeny virus can be reproduced when the larger amount of virus is inoculated. First of all, such a progeny virus which was reproduced under the presence of No.799 was examined in point of view of the resistance to No.799. The parent virus, No.799 sensitive Mahoney strain of polio virus, was inoculated into tubes in which the monosheet of host cells had been established, and in minutes, 10^{-3}M of No.799 was added into the tubes. As inoculaum size of the Mahoney strain, $10^{7.5}$ $TCID_{50}$ was employed. After the complete cell-degeneration, the mixture cells-culture fluid was frozen and thawed five times, and then centrifuged at 3000 rpm for 10 minutes. The supernatant was employed as a material of the progeny virus. The $TCID_{50}$ of both No.799 sensitive parent Mahoney and the progeny virus was determined by usirg both the No.799 free ordinary maintenance medium and the 10^{-3}M of No.799 containing maintenance medium. The experimental results are shown in Table 4.

Table 4

Development of No.799 resistant Mahoney Virus

Viral materials	$TCLD_{50}$ ($-Log_{10}$) No.799 free medium	No.799 containing medium
Parent virus	7.5	3.5
Progeny virus	7.5	7.5

As can be seen in Table 4, the No.799 resistant virus can be developed even after the first passage in the presence of

No. 799.

(b) Cross-resistance between the Guanidine-resistant Mahoney and the No. 799-resistant Mahoney.

At the next stage, the cross-resistance between the guanidine-resistant Mahoney and the No. 799-resistant Mahoney was examined.

The experimental results are shown in Table 5.

Table 5

Cross-resistance between Guanidine-resistant Virus and No. 799-resistant Virus.

Viral Materials	$TCID_{50}(-\log_{10})$		
	Guanidine and No. 799 free medium	Guanidine containing medium	No. 799 containing medium
Guanidine resistant Mahoney strain	7.0	7.0	4.5
No. 799 resistant Mahoney strain	7.5	5.0	7.5
Parent Mahoney	7.5	5.0	3.5

As can be seen in Table 5, the cross-resistance between guanidine-resistant virus and No. 799-resistant one could not be found. These results suggest that the mechanism of the antiviral effect of No. 799 may be different from that of guanidine.

(c) Time-course of the intracellular multiplication of the Guanidine-resistant Mahoney strain.

The time necessary to develop the guanidine resistant polio virus was determined.

$10^{6.5}$ $TCID_{50}$ of the guanidine sensitive Mahoney strain was inoculated into assay tubes in which the monosheet of

host cells had been established, and these tubes were incubated at 37℃ for one hour. After incubation, the medium was removed, and the cells were washed three times with PBS, and then the 10^{-3}M of guanidine nitrate containing maintenance medium was added into the tubes. At various intervals after the addition of guanidine, the culture fluid was removed from the tubes, and the resistance of the progeny virus was estimated.

The experimental results are shown in Table 6.

Table 6

Time-course of Development of Guanidine-resistant Mahoney Virus

Hours at which progeny virus was collected (hours after addition of guanidine)	$TCID_{50}$ (log_{10}) Guanidine free medium	Guanidine containing medium
6	1.5	C.I.※
8	2	C.I.
10	2	C.I.
12	2	C.I.
16	4.5	3.5
21	4.75	3.5
34	4.75	5
46	5.5	5.5

※ Complete inhibition.

From the results shown in Table 6, it may be said that the complete development of the guanidine resistant polio virus was found from twenty one hours to thirty four hours after the addition of guanidine. These results suggest that this

mutant may be produced by the mechanism of selection.

D. Effect of Guanidine plus Ethionine on Poliomyelitis Virus.

To find more effective compounds than guanidine, the responsible investigator synthesized many types of compounds, and found No.799 from the screening tests as to their antiviral effect. In another type of experiments, the responsible investigator found as described in the quarterly progress report inclusive dates 15 February 1961 to 14 May 1961 that the effect of guanidine was potentiated by the addition of ethionine in spite of the lack of the antiviral effect of the latter compound. Thus, the effect of guanidine plus ethionine on virus was further studied.

After the establishment of the monosheet of host cells in assay tubes, both of guanidine and ethionine in 10^{-3}M in final dilution respectively were added into tubes, immediately later, various dilutions of a viral material were inoculated into these tubes. After incubation at 37℃ for 10 days, $TCID_{50}$ of the control and the treated group was estimated. In the control, PBS was used instead of guanidine and ethionine.

The experimental results are shown in Table 7.

Table 7.

Effect of Guanidine plus Ethionine on Polio Virus.

Viral materials	$TCID_{50}$(-log)		
	control	guanidine containing medium	guanidine and ethionine containing medium
Mahoney strain	7.5	4.0	2.5
	6.5		

MEF, strain	6.5	4.0	
	7.0		3.5
Saukett strain	8.0	5.5	
	7.5		4.0

As can be seen in Table 7, the effect of guanidine plus ethionine was greater than that of guanidine alone. As to the antiviral effect, W.W.Ackermann reported that ethionine showed the inhibitory effect on the growth of polio virus, but Syverton denied the effect of this compound from the results of his minute experiments. Syverton reported that ethionine was inhibitory only when it was employed in such a large amount to inhibit the respiration of host cells. The responsible investigator also showed as described in the quarterly progress report inclusive dates 15 February 1961 to 14 May 1961 that the nontoxic dose of ethionine was ineffective on the growth of polio virus in host cells. As shown in Table 7, ethionine potentiates the antiviral effect of guanidine in spite of the lack of the antiviral property of itself. This effect is not of chemotherapeutic synergism, because ethionine does not possess antiviral property.

At present time, the reason why ethionine potentiates the effect of guanidine is not clear yet, but these findings will give a new way for the search of antiviral agents.

E. On An Inhibitory Factor Produced in Normal Culture Fluid.

Concerning the virus inhibitory agents, which are produced with the infection of a viral material into host cells, a lot of attempts have so far been carried out. Issacs and his coworkers reported the production of Interferon, and

Enders and Ho found a virus inhibitory agent from the culture infected with measles and inactivated polio viruses and Nagano and his workers reported that vaccinia virus produced virus inhibitory factor in rabbits. On the way of the investigation of Interferon and Interferon like substances, the responsible investigator found that the cell-culture-fluid of the cells which are not infected with any viral material inhibited the development of plaques of polio virus. From Isaacs' findings concerning Interferon upto now, many researches concerning the production of Interferon like substances have been carried out, but all these attempts have been tried using cells or tissues infected with virus. The present work of the responsible investigator and the coworkers cocerns with an inhibitor obtained from normal cell-culture-fluid.

(a) Preparation of inhibitor: After the establishment of the monosheet of employed cells, the growth medium was removed, then the cultured cells were washed three times with PBS, and the maintenance medium was added into the culture-bottle. This bottle was placed at 37℃ for various periods without any exchange of medium. After the incubation from 2 days to 21 days the culture fluid was removed and the fluid was centrifuged at 3000 rpm for ten minutes, and the supernatant was collected as a material of inhibitor.

(b) The system for the assay of an inhibitor: 0.5cc. of the collected culture-fluid was added into a bottle, in which the monosheet of an employed cells had been established, and 0.5cc. of a viral material was inoculated. After incubation at 37℃ for one hour, the mixture of an inhibitor and

viral dilution was removed, then the cells were washed three times with PBS. After that, 0.5cc. of an inhibitor was added in to the bottle, then immediately later, agar overlay was placed. After incubation at 37C for three days, the numbers of plaques were counted.

(c) Effect of inhibitor on each of types of polio virus.

The effect of this inhibitor on each of types of polio virus was examined. The experimental results are shown in Table 8.

Table 8

Effect of Plaque Formation of Each Type of Polio Virus

Viral material	PFU/ml		Decreased Percentage %
	Control	Treated	
Mahoney	4.3×10^7	2.4×10^7	44.2
MEF_1	3.9×10^7	2.1×10^7	46.2
Saukett	6.0×10^7	3.1×10^7	48.3

From these results, it may be said that this inhibitor is effective on all of three types of polio virus and so it is not a sero-specific inhibitor like an antibody.

(d) Participation of serum in the production of an inhibitor.

The above results suggest that this new inhibitor must be quite different from sero-specific antibodies of polio virus, but there are other remaining problems to be solved, since serum contains many nonserospecific inhibitors like α- and β-inhibitors, properdin and so on. Thus, the responsible investigator studied the production of this inhibitor in the culture by using the synthetic medium without any supplement of serum.

After the establishment of the monolayer of HeLa cells, the growth medium was removed. The cellsheet was washed three times with PBS, and then the Eagle's synthetic medium supplemented with 0.8% polyvinylpyrrolidone(PVP) was added into the culture bottle.

After incubation at 37℃ for ten days, the culture fluid was removed from the bottle, and centrifuged at 2000rpm for ten minutes, and the supernatant was used as a tested material.

The experimental results are shown in Table 9.

Table 9

Effect of An Inhibitor produced in the Cell-culture by using Synthetic Medium without any Supplement of Serum

	Plaque-numbers	Decreased Percentage of Plaque
Ordinary YLA medium added with 5% bovine serum	46	
HeLa-cultured fluid(Eagle's medium with 0.8% PVP) incubated at 37℃ for ten days	28.5	38.9
Eagle's medium with 0.8% PVP incubated in a tube, which does not contain, at 37℃ for ten days	41.5	9.8

The results shown in Table 9 indicate that this inhibitor can be produced from the culture of HeLa-PVP supplemented Eagle's medium.system,butthe PVP supplemented Eagle's medium,

which was incubated at the same temperature for the same days in a tube in which no cell was inoculated, did not show any inhibitory effect. This suggests that the new inhibitor can be produced even in a protein free environment and is not consisted of type-specific antibodies or nonspecific inhibitors contained in serum, and for the production of this new inhibitor the presence of cells should be essential.

(e) Participation of Holland's Receptor in the production of an inhibitor.

Holland has reported about the presence of a receptor in the cells sensitive on polio virus. This receptor combines with viral particles and inacivates the infectivity of the particles. Thus, the problem whether the new inhibitor is just the same to Holland's receptor released from host cells or not is still remained to be solved. The responsible investigator examined this point.

The experimental results are shown in Table 10.

Table 10

Comparison of the Effect of an Inhibitor with That of Holland's Receptor.

	Numbers of Plaques		Decreased Percentage
	Control	treated	%
Inhibitor from KB culture	46	28.6	38.8
Holland's receptor	30.6	29.0	5.2

From the results shown in Table 10, it may be said that the new inhibitor should be quite different from Holland's receptor, because the receptor did not inhibit the develop-

ment of plaque, while the inhibitor showed clearly the inhibition of plaque-formation.

(f) Chemical natures of a new viral inhibitor.

From the experimental results described in the above, it was assumed that this inhibitor is a newly found viral inhibitory agent which is different from all of Interferon, Interferon like substances, sero-specific antibodies, non-specific inhibitors in serum and Holland's receptor. Thus, the responsible investigator furthermore investigated about the chemical natures of this new viral inhibitor.

The experimental results are shown in Table 11.

Table 11

Chemical Natures of A New Viral Inhibitor.

Treatments	Numbers of Plaques	Decreased Percentage %
Control	86	
Original culture fluid of HeLa cells at 37C for ten days	49.5	42.3
Heated at 56C for 30 minutes	49	43
Heated at 100C for 10 minutes	54.5	36
Frozen and thawed five times	69.5	19.2
Dialysed at 4C for 24 hours	44	48.8
Digested with 0.25% Trypsin	41	52.3
Digested with 100γ of RNA-ase	30	65.1

From the results shown in Table 11, it may be considered that this new viral inhibitory agent is a high molecular substance which is stable against heat-inactivation, but not of simple protein or RNA.

The viral inhibitory potency of this new inhibitor is not so high at present, and so the responsible investigator now is studying the isolation and the purification of this agent.

5. Conclusion Drawn.

From the above experimental results, the following points were found.

(1) 107 compounds were synthesized during the past one year and received the screening tests as to their antiviral effect.

(2) Among these 107 comounds, the following two compounds, 4-Amino-6-morpholinyl-s-triazine-2-carboxyguanidide and 4-Amino-6-isopropylamino-s-triazine-2-carboxyguanidide, showed the remarkable effect on polio virus. The former compound was called No.799 and the latter compound is synthesized again because the first investigative sample was used up.

(3) No.799 was more effective on polio virus than guanidine was, and possessed more broad antiviral spectrum.

(4) No.799 was effective on such entero viruses as three types of polio virus, ECHO type-6, coxsackie A-9 and coxsackie B-5, measles virus and influenza in mice.

(5) From the investigation on the site of action of No. 799, it was made clear that this compound inhibits the intracellular reproduction of polio virus, particularly the latent phase of the viral multiplication, because this compound did not inhibit the viral adsorption onto cells and the viral release from host cells, and moreover it did not possess any direct viral inactivating action. The effect of No. 799 was

shown most clearly when it was added to culture at the latent phase after the viral penetration had been established.

(6) The progeny polio virus which was reproduced under the presence of No.799 showed the resistance to the antiviral effect of No.799. But the cross-resistance between the guanidine resistant virus and the No.799 resistant virus could not be found. These results suggest that the mode of action of No.799 should be different from that of guanidine.

(7) From the studies on the time-course of the development of guanidine resistant polio virus, it was shown that the incubation time of about twenty-thirty hours after the addition of guanidine to viral culture is necessary to obtain a complete guanidine resistant mutant of polio virus. This suggests that the development of guanidine resistant polio virus virus must be due to a selection mechanism.

(8) From the investigation as to the potentiation of guanidine with ethionine, the effect of guanidine plus ethionine was greater than that of guanidine alone. The potentiating effect of ethionine to guanidine is not so-called chemotherapeutic synergism, because the nontoxic concentration of ethionine does not show any effect on virus. This effect of ethionine must be of new types of drug-potentiation.

(9) On the way of the investigation of Interferon and Interferon like substances, it was found that the cell culture without any infection of virus produced a viral inhibitory substance. This agent was quite different from Interferon, Interferon like substance, sero-specific antibodies, non-specific inhibitors in serum and Holland's receptor.

(10) This new viral inhibitory substance was stable against heat-inactivation, trypsin-digest and RNA-ase, and was not dialysed. Thus, it was assumed that this new viral inhibitor should be a high molecular substance, but not of simple proteins or ribonucleic acid. The findings of this new viral inhibitor will suggest a way to find highly effective antiviral biological substances.

6. Implication of Conclusion.

At present, any highly effective antiviral chemotherapeutic drug on not only such neurotropic viruses as poliomyelitis and Japanese B Encephalitis but any other small animal virus has not been found yet. About three years ago, the responsible investigator and the coworkers found that guanidine showed the effect on the multiplication of both poliomyelitis and measles viruses. On the basis of the findings, the search to find more effective compounds than guanidine has been carried out. During the past one year, from 15 February 1962 to 14 February 1963, 107 compounds were synthesized for this purpose, and No. 799, 4-Amino-6-morpholinyl-s-triazine-2-carboxyguanidide, was found as the more highly inhibitory compound than guanidine, and moreover this compound possesses more broad antiviral spectrum than guanidine, in other words, guanidine was completely ineffective on influenza in mice, while this compound showed the therapeutic effect on the mice infected with influenza virus. The effect of this compound on respiratory viruses will be investigated in more detail. At any rate, the findings of the antiviral effect of No.799 suggest that the search of

the related compounds of the bases of nucleic acid must be of the most promising ways to find highly effective antiviral agents.

Since the discovery of Interferon by Isaacs, many attempts to find viral inhibitory agents from the culture infected with virus have been carried out, and a lot of viral inhibitory factors have been found. These factors, however, did not show satisfactory effect to be recognized as a chemotherapeutic agent and moreover it was rather difficult to concentrate these agent to increase the antiviral potency, because all of them were very sensitive to heat-inactivation. On the way of the investigation of Interferon like sunstances, the responsible investigator found a new viral inhibitory agent, which was produced from the cells which was not infected with any virus, and this agent was found as a very stable one against heat-inactivation. This nature must be of benefit to concentrate or purify this agent.

From the investigation concerning the effect of guanidine plus ethionine, it was found that the potentiating effect of ethionine on guanidine is clearly observed and is not of so-called chemotherapeutic synergism. The findings of the potentiation with a second substance to an antiviral agent will give a way to find antiviral agents.

In the field of virology, chemotherapy and biochemistory, so satisfactory knowledge as to the mechanism of the multiplication or the replication of virus has not been obtained yet. If any information as to such a point is obtained, it will be very much useful to conceive new types of compounds for the

search of antiviral agents. During the past several years, a lot of work to solve the replication mechanism have been carried out by using such modern techniques as tracer-technique, density gradient centrifugation, autoradiography and so on, but specific metabolic pathway essential for viral replication could not solved so clearly. Even at present time, the mechanism for viral replication is still a mysterious spinx. The responsible investigator think, some specific key such as a specific compound blocking viral replication or a specific mutant virus will be necessary to open a door of viral replication mechanism. In this point of view, the findings of the effect of No. 799 and the resistant polio virus to guanidine and No. 799 will be very valuable tools to obtain fundamental knowledges of the mechanism of viral replication.

7. Positive or Negative Corroboration.

During the past one year, 107 compounds were synthesized and screened as to their antiviral effect. Among these 107 compounds, No. 799, 4-Amino-6-morpholinyl-s-triazine-2-carboxyguanidide, was found as a more effective compound than guanidine, and this compound was effective on influenza in mice, too, while guanidine was completely ineffective on the virus. This finding must surely be a very valuable milestone to find a highly effective antiviral chemotherapeutic drug.

On the way of the investigation of Interferon like substances, a new viral inhibitor produced from cell-culture which is not infected with any virus was found. The chemical nature of this new inhibitor is clearly different from Interferon and Interferon like substances, and particularly the

heat-stability of this new inhibitor must be of benefit to concentrate or purify it highly. The finding of this inhibitor will be a new way to find antiviral agent.

The investigation of the effect of guanidine plus ethionine showed that the effect of the combined substance was greater than that of guanidine alone, and moreover this potentiation of ethionine is not caused by so-called chemotherapeutic synergism. This new type of drug-potentiation will be a useful tool to find antiviral agents.

In the investigation of drug-resistant mutants, the two mutants, guanidine resistant polio virus and No.799 resistant one. These mutants will be very much usefull to investigate the mechanism of viral replication in host cells.

8. Contribution to Theory.

In the research field of nucleic acid antagonists, both of cancer cells and microorganisms have been employed as the assay system, and a lot of nucleic acid antagonists have been found. In all viruses, nucleic acid roles the most important part for the infectivity and it was considered by almost all research workers that the most essential problem in viral replication must be the biosynthesis of viral nucleic acid and some effective antiviral agents should be found by blocking some processes of viral nucleic acid-biosynthesis. For this purpose, many nucleic acid antagonists, which were found by using cancer cells or microorganisms as the assay system, have been employed. So effective antiviral agents, however, have not been found from such trials. The responsible investigator have thought, the nature of viral nucleic acid and its replication in host

cells must be quite different from that of nucleic acids of cancer cells or microorganisms. Accordingly, the nucleic acid antagonists, which were found from the assay using cancer cells or microorganisms, must be specific to such cell-lines as cancer ceffs or microorganisms, but not specific to viral nucleic acid. Indeed, the responsible investigator had examined the effect of such common nucleic acid antagonists as alkylating agents, 6-aminopurine, 2,6-dimercapopurine, benzimidazole and so on. These compounds, however, did not show so remarkable effect on the viral growth. On the basis of such fundamental knowledges obtained in the Institute of the responsible investigator, many new types of the homologues of the bases of nucleic acid were synthesized and screened as to their effect. Among these compounds, 4-Amino-6-morpholinyl-s-triazine-2-carboxyguanidide(No.799) was found as the most promising compound. This compound is a new homologue of the bases of nucleic acid, and has been found on the basis of an idea obtained from the fundamental knowledges as to the antiviral agents in this Institute. The finding of No.799 will show a new way to investigate new types of nucleic acid antagonists specific to viral nucleic acid and its biosynthesis.

At the next, it is a well known fact in the chemotherapeutic field that the addition of some second compound shows a synergic action to a chemotherapeutic drug, if this second compound possesses the effect similar to that of the drug. On the contrary, the responsible investigator and the coworkers found a quite different phenomenon from such a synergism about sixteen years ago. They found that some compounds, which

did not possess any therapeutic activity, can potentiate the therapeutic effect of a chemotherapeutic drug. For example, it was found by the responsible investigator that the therapeutic effect of organic arsenicals on treponema and trypanosoma could be potentiate by the addition of such amino acids as glutamic acid and histidine. As described in this report, ethionine can potentiate the antiviral effect of guanidine in spite of the lack of the antiviral effect. This finding is a contribution to the theory of the responsible investigator concerning the second potentiating component for a chemotherapeutic drug. Along this direction, the search as to any other potentiating components will be carried out.

For the investigation of high molecular viral inhibitory agent, the responsible investigator has studied Interferon like viral inhibitory agents. On the way of this investigation, the following idea was thought by the responsible investigator. If Interferon or Interferon like substances are for natural protection of a body against the invasion of pathogenic microorganisms, some resemble factors with Interferon or Interferon like substances will be even in a normal healthy body or tissues, and if it is the case, it will be shown in tissue culture system. In fact, it was been often observed that some tubes show cytephatic effect but some other tubes are completely negative in the appearance of cell-degeneration if very high dilution of a viral material is inoculated. Many research workers have explained this phenomenon as host-resistance or unsyncronization of host-sensitivity to viral infection. The responsible investigator, however, think

that such a phenomenon might be caused by a viral inhibitor produced from host cells, and this inhibitor might be produced at any time from normal cells for the protection of cells themselves. On the basis of such an idea, the responsible investigator carried out the investigation of nonspecific viral inhibitor produced from host cells which were infected without any virus. The viral inhibitor described in this report was a great contribution for such an idea of the responsible investigator. The responsible investigator think, such an inhibitor produced from normal cells may be role a great part for the development of a pre-condition for the appearance of the therapeutic effect of a drug against the infection of pathogenic microorganisms. If this inhibitor is concentrated or highly purified, it will be very useful to find antiviral agents.

As described in this final report, a lot of fundamental knowledges to conceive new types of compounds were obtained. During the past one year, many research problems have been carried out under the contract with U.S.Army R. and D.Group (Far East). This is the greatest happiness of the responsible investigator and all members of the staff of this Institute. On the basis of the findings obtained during the past one year, the search will be carried out furthermore. The work to find highly effective antiviral agents must be terribly difficult, while our progress is rather slow. But we will carry out this work steady and steady. If we do so, we shall arrive at the goal soon or later, we believe so.

AD Div 16/2

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Final rept No. 2, 15 Feb 62 - 14 Feb 63. 35 p. incl. illus. tables.
(Contract DA 92-557-FEC-34625) Unclassified report

The purpose of this work is to find effective antiviral agents on poliomyelitis and Japanese B Encephalitis viruses, and the findings obtained in this work will be milestone to find antiviral compounds on any other small animal viruses. For this purpose, 107 compounds were synthesized and screened as to their effect. Among them, it was found that compound No. 799, 4-Amino-6-(N-morpholinyl)-s-triazine-2-carboxyguanidide, was highly inhibitory on the multiplication of polio, ECHO and coxsackie viruses, and the site of action of compound.

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2. Chemotherapeutic Agents - Poliomyelitis Virus

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